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Sym28 and *Sym29*, Two New Genes Involved in Regulation of Nodulation in Pea (*Pisum sativum* L.)

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Abstract

Seeds of *Pisum sativum* L. cv Frisson were treated by ethyl methane sulphonate to create mutations affecting nodulation. M_2 plants were grown in high nitrate conditions and putative nitrate-tolerant nodulation (Nts) mutants were selected. Mutant phenotypes were confirmed in M_3, M_4, M_5 and M_6 generations. Eight new Nts mutant lines, showing a supernodulating (Nod^{++}) character when grown without nitrate, were identified. All Nod^{++}Nts mutations were shown to be controlled by single recessive genes and resulted from mutation events at two different loci, *sym28* and *sym29*. Complementation analyses with previously-described mutant lines, P64 from cv Frisson and Nod3 from cv Rondo, identified three genes controlling Nod^{++}Nts character in pea. Grafting experiments showed that Nod^{++}Nts phenotype was associated with the shoot genotype in all cv Frisson-derived mutant lines, while the root control of Nod^{++} phenotype was confirmed for Rondo-derived line Nod3.

Keywords: *Pisum sativum*, mutagenesis, supernodulation, nitrate-tolerant nodulation, *Rhizobium leguminosarum*

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1. Introduction

Nodulation in the legume-*Rhizobium* symbiosis is controlled by a signal and response mechanism that suppresses nodule emergence in the younger part of the root system once a critical number of nodules has been formed (Gresshoff, 1993a). This phenomenon occurred in the presence (inhibition of nodulation by nitrate) and in the absence (autoregulation or feedback control of nodule number) of nitrate and is mostly controlled by the host symbiont (Gresshoff, 1993a). Plants which are defective in autoregulation and which show a supernodulation (Nod^{++}) character have been selected in soybean (Carroll et al., 1985; Gremaud and Harper, 1989; Akao and Kouchi, 1992), pea (Jacobsen and Feenstra, 1984; Duc and Messager, 1989), common bean (Park and Buttery, 1988), faba bean (Duc, 1995) and in *Medicago truncatula* (Gaertn.) (Sagan et al., 1995). Split root and grafting experiments showed that autoregulation was systemic in soybean (Kosslak and Bohlool, 1984; Delves et al., 1986; Olsson et al., 1989; Cho and Harper, 1991; Francisco and Harper, 1995), alfalfa (Caetano-Anollés and Gresshoff, 1991), common bean (Buttery and Park, 1990), faba bean (Duc, 1995) and pea (Duc and Messager, 1989). Supernodulation was controlled by the shoot genotype in soybean (Delves et al., 1986, Cho and Harper, 1991; Francisco and Harper, 1995), common bean (Buttery and Park, 1990), faba bean (Duc, 1995) and in the pea line P64 of cv Frisson (Duc and Messager, 1989). However, Postma et al. (1988) described a root control of supernodulation in the pea mutant Nod3 of cv Rondo.

These supernodulating genotypes all showed a nitrate tolerant nodulation (Nts) character (Jacobsen and Feenstra, 1984; Carroll et al., 1985; Gremaud and Harper, 1989; Park and Buttery, 1988; Duc and Messager, 1989; Akao and Kouchi, 1992; Duc, 1995, Sagan et al., 1995) suggesting that the regulation of nodulation by internal (autoregulation) and external (nitrate) signals shares a common mechanism. Genetic analyses identified one gene controlling nodule number in soybean (Gresshoff, 1993b) and common bean (Park and Buttery, 1988). Complementation analyses have, so far, not yet been performed in pea, where several supernodulating lines have been described (Jacobsen and Feenstra, 1984; Duc and Messager, 1989).

In this paper, we describe the selection of eight new $NtsNod^{++}$ mutant lines of pea cv Frisson. Complementation analyses identified two genes among the selected mutants and three in pea. Reciprocal grafting experiments showed that Nts Nod^{++} phenotype was controlled by the shoot genotype in all cv Frisson-derived mutant lines, while the root control of supernodulation was confirmed in cv Rondo-derived mutant Nod3.

2. Material and Methods

Plant material and Rhizobium strain

Pisum sativum (L.) cv Frisson was used as the parental line for the mutagenesis treatment and as wild-type control line for other experiments. The supernodulating and nitrate-tolerant nodulation ($Nod^{++}Nts$) mutant P64, selected from cv Frisson (Duc and Messager, 1989), was used as a supernodulating control line in mutant selection experiments, complementation analyses and grafting experiments. P64 was denoted in the original description as 191F (Duc and Messager, 1989). The supernodulating mutant line Nod3, selected from pea cv Rondo (Jacobsen and Feenstra, 1984) (provided by E. Jacobsen, University of Groningen, Haren, The Netherlands) was used in complementation analyses and grafting experiments. The nodulation mutants (Nod^- or $Nod^{+/-}$) P2, P54, P57 from cv Frisson (Duc and Messager, 1989; Sagan et al., 1994) and DK24 from cv Finale (provided by K.C. Engvild, RISO National Laboratory, Roskilde, Denmark) were used in grafting experiments. Previous studies showed that mutant phenotypes were controlled by the root genotype in P2, P54, P57 and Nod3 (Duc and Messager, 1989; Postma et al., 1988), while supernodulation in P64 was controlled by the shoot (Duc and Messager, 1989).

In all experiments, plants were inoculated with the *Rhizobium leguminosarum* bv *viciae* strain 1007 (provided by N. Amarger, Laboratory of Microbiology, INRA, Dijon, France) which forms effective nodules on pea. Unless mentioned, plants were inoculated at sowing time and grown in glasshouse conditions, on sterile pozzolana (granules of volcanic rock) substrate as described by Duc and Messager (1989).

Mutagenesis treatment and screening procedure

Ten thousand seeds of pea cv Frisson were incubated in 0.1% ethyl methane sulphonate (EMS) for 6 h, washed under running water and sown in greenhouse as described previously (Duc and Messager, 1989). M_2 progenies of resulting plants were harvested individually.

1472 M_2 families (about 12 seeds per M_2 progeny) were sown and inoculated with *Rhizobium*. Plants were watered daily with a nutrient solution containing 10 mM of nitrate (Duc and Messager, 1989) and visually screened for nodulation phenotype at early flowering stage. Plants with a high number of nodules compared to cv Frisson controls were selected and transferred in pots to produce M_3 seeds.

The M_3, M_4, M_5 and M_6 progenies (12 plants per progeny) from the putative Nts mutant lines were tested again both in N-free and 10 mM nitrate conditions

(Duc and Messager, 1989) to check for the Nod^{++} (supernodulation: high number of nodules on plants grown without nitrate) and Nts (nitrate tolerant nodulation) characters respectively, and for the genetic stability of their phenotypes.

Genetic analyses

Crosses were made between the selected mutants and the parental genotype cv Frisson. All plants in each F_1 and a sample of F_2 plants (average of 400 plants) from each mutant x cv Frisson cross were tested for nodulation phenotype in N-free growing conditions. Crosses were then made between selected mutants, in a half diallel design, and F_1 progenies were tested in N-free conditions. One mutant line of each complementation group was crossed with the previously-identified Nod^{++} mutant lines P64 (Duc and Messager, 1989) and Nod3 (Jacobsen and Feenstra, 1984), and F_1 progenies were tested for nodulation phenotype in N-free conditions.

Phenotypic variation between mutants for supernodulation and nitrate tolerance characters

Seeds of cv Frisson and of the mutant lines were surface-sterilized for 20 min in 7% (w/v) calcium hypochlorite and thoroughly rinsed in sterile distilled water. Plants were grown in modified Leonard jars using terragreen (Soil Condition, Laporte SA, USA) as a substrate and an N-free, 5 mM or 10 mM nitrate nutrient solution as described by Sagan et al. (1993b). Six Leonard jars (one plant per jar) for each genotype x nitrate combination were used. Plants were inoculated one day after planting. Plants were examined one month after inoculation and nodule number and shoot dry matter per plant were scored.

Grafting experiments

Reciprocal grafts were made on 7-day-old axenically-grown seedlings between the primary scale and the first leaf. Lateral shoots appearing below the graft were removed daily after grafting. Three experiments were done: (i) reciprocal grafts between each selected mutant and cv Frisson, screening in N-free and 10 mM nitrate growing conditions; (ii) reciprocal grafts between each selected mutant and the nodulation mutants, screening in N-free an 10 mM nitrate growing-conditions; and (iii) reciprocal grafts in a diallel design between P64, Nod3, cv Frisson and the selected mutant P88, screening in N-free growing conditions. In each experiments, self-grafts and non-grafted plants were used as controls.

Seedlings were inoculated one day after grafting and eight plants of each grafting combination were screened at flowering stage. Phenotypes were determined visually in experiments 1 and 2. The number of nodules per plant was scored in experiment 3.

Statistical analyses

χ^2 analyses were performed on F_2 segregation (wild-type phenotype:mutant phenotype) data. Analyses of variance were performed on nodule number and shoot dry matter data and means were classified according to the Newman-Keuls test.

3. Results

Selection of mutants

About 17600 M_2 plants (1472 M_2 progenies) were screened for the nitrate-tolerant nodulation (Nts) character. Putative mutants were selected and transferred in pots to produce M_3 seeds. Among these putative mutants, the Nts phenotype of eleven lines belonging to eight families was confirmed in the M_3 generation. These eleven lines also showed a Nod^{++} character when grown without nitrate and the association Nts-Nod^{++} phenotypes was confirmed in M_4, M_5 and M_6 generations. There was no difference of phenotype between progenies of mutant lines of the same M_2 family and only one filiation was kept after the M_4 generation. The mutation frequency for the Nts-Nod^{++} character was 1 mutant for 1600 M_2 plants or 1 mutant line for 184 M_2 family. Table 1 lists the mutant lines and Fig. 1 shows the root systems of the parental line cv Frisson and of mutant P88.

Genetic analyses

All F_1 plants from crosses of the mutants with cv Frisson showed a wild-type ([+]) phenotype. The F_2 plants from these crosses showed a segregation in wild-type:supernodulation phenotypes not deviating from 3:1 (Table 1), which is, in all cases, in agreement with the hypothesis of monogenic and recessive mutations.

Diallel crosses between the mutants led to wild-type and supernodulating F_1 progenies (Table 2). Two complementation groups were identified. Complementation group 1 comprised only one mutant (P77) while group 2 comprised seven mutants (P87, P88, P89, P90, P91, P93, P94) (Table 2). Complementation

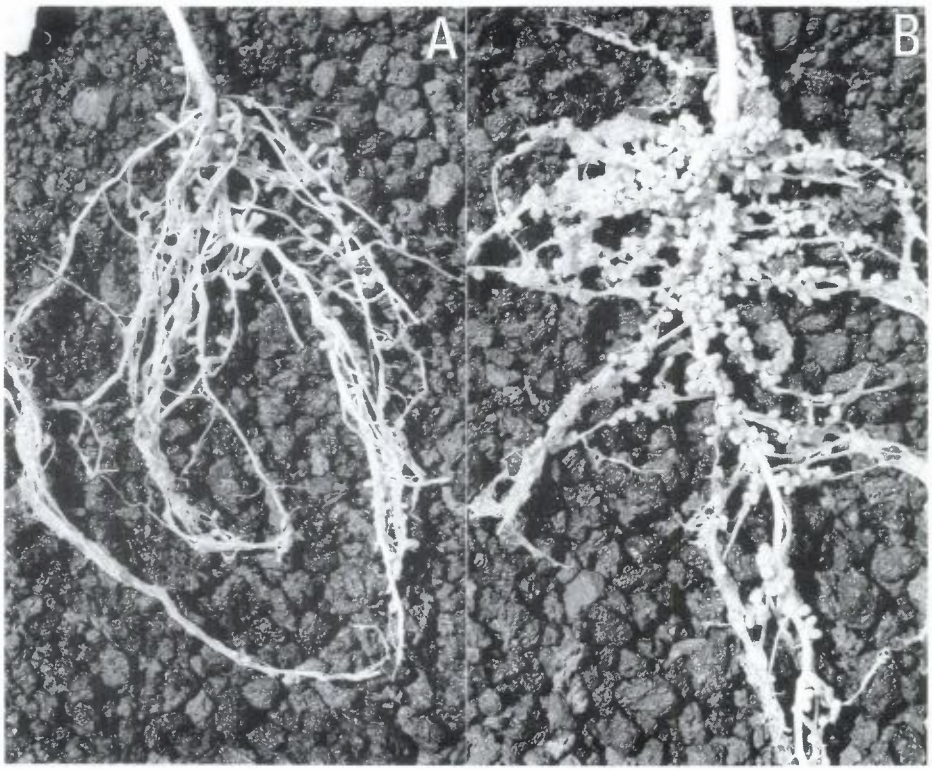

Figure 1. Root system of *Pisum sativum* L. cv Frisson (A) and of supernodulating mutant P88 (B).

analyses of P64, Nod3, P77 and P88 showed that P64 and P77 were allelic, while Nod3 and P88 belonged to two other complementation groups (Table 3). P77 also showed a stem fasciation character as previously described for mutant line P64 (Duc and Messager, 1989). F_1 hybrides of P77 with cv Frisson all had a wild-type phenotype. F_2 progenies of these crosses segregated 3:1 wild-type:supernodulation-fasciation and no dissociation of the supernodulation and fasciation characters was detected among more than 500 F_2 plants. Crosses between P64 and P77 led to F_1 plants showing supernodulation and fasciation characters. This is in agreement with the hypothesis that the same recessive gene controls supernodulation and fasciation in P64 and P77.

Mutants of complementation group 2 all showed a short internode character and no dissociation was detected between supernodulation and the short internode character among progenies of crosses with these mutant lines. This is

Table 1. Nodulation phenotype of selected mutant lines and F_1 and F_2 progeny phenotypes of crosses between mutant lines and cv Frisson.

Mutant line	Nodulation phenotype	Cross: Mutant x cv Frisson Number of plants of mutant and wild-type phenotypes in F_1 and F_2 progenies				
		Generation	Mutant phenotype	Wild-type phenotype	χ^2 of 1:3	P
P77	Nod^{++}Nts	F_1		16		
		F_2	131	440	1.29	>0.2
P87	Nod^{++}Nts	F_1		38		
		F_2	134	390	0.05	>0.9
P88	Nod^{++}Nts	F_1		26		
		F_2	145	446	0.07	>0.5
P89	Nod^{++}Nts	F_1		25		
		F_2	77	247	0.26	>0.5
P90	Nod^{++}Nts	F_1		12		
		F_2	55	182	0.41	>0.5
P91	Nod^{++}Nts	F_1		35		
		F_2	35	129	1.17	>0.3
P93	Nod^{++}Nts	F_1		10		
		F_2	83	243	0.04	>0.5
P94	Nod^{++}Nts	F_1		21		
		F_2	132	387	0.05	>0.5

also in agreement with the hypothesis that the same recessive gene controls supernodulation and short internode characters in these lines.

Phenotypic variation between mutants for hypernodulation and nitrate tolerance characters

Mutant lines were inoculated with *Rhizobium* strain 1007 and grown in Leonard jars in presence of 0, 5 or 10 mM of nitrate. The number of nodules and the shoot dry matter were scored 30 days after inoculation.

Table 2. Number of crosses between the eight selected mutant lines and nodulation phenotype of F_1 progenies.

	Parental lines							
Parental lines	P77	P87	P88	P89	P90	P91	P93	P94
P77	Nod^{++}	2[+]	4[+]	5[+]	4[+]	4[+]	5[+]	5[+]
P87		Nod^{++}	4 Nod^{++}	1 Nod^{++}	2 Nod^{++}	4 Nod^{++}	9 Nod^{++}	3 Nod^{++}
P88			Nod^{++}	2 Nod^{++}	11 Nod^{++}	5 Nod^{++}	5 Nod^{++}	4 Nod^{++}
P89				Nod^{++}	1 Nod^{++}	5 Nod^{++}	9 Nod^{++}	1 Nod^{++}
P90					Nod^{++}	4 Nod^{++}	5 Nod^{++}	5 Nod^{++}
P91						Nod^{++}	5 Nod^{++}	4 Nod^{++}
P93							Nod^{++}	7 Nod^{++}
P94								Nod^{++}

Mutant lines whose cross leads to supernodulating (Nod^{++}) progenies are allelic, while those leading to wild-type ([+]) progenies belong to different complementation groups.

Table 3. Number of crosses between selected lines P77 and P88, and the previously-described supernodulating mutants P64 and Nod3, and nodulation phenotypes of F_1 progenies.

	Parental lines			
Parental lines	P64	P77	P88	Nod3
P64	Nod^{++}	4 Nod^{++}	4 [+]	7 [+]
P77		Nod^{++}	4 [+]	4 [+]
P88			Nod^{++}	9 [+]
Nod3				Nod^{++}

Mutant lines whose cross leads to supernodulating (Nod^{++}) progenies are allelic, while those leading to wild-type ([+]) progenies belong to different complementation groups.

A significant effect of genotype and of the genotype x nitrate interaction on nodule number per plant was detected through variance analysis, while no significant effect of nitrate was found (Table 4). Nodule number data were consistent with the previously visually-determined phenotypes of the

mutants. A significantly greater number of nodules was recorded for the mutant lines which showed an average of 697 nodules per plant when grown without nitrate, while cv Frisson had 178 nodules (Fig. 2). Cv Frisson showed a 24 and 32% decrease in nodule number per plant when grown with 5 and 10 mM nitrate respectively (Fig. 2). Selected mutants and P64 all had significantly higher number of nodules compared to cv Frisson when grown with nitrate, and an average of 523 and 707 nodules per plant was found for 5 and 10 mM nitrate respectively (Fig. 2). This confirms the previously-described Nts phenotype of these lines. However, there were differences between complementation groups and among lines of a same complementation group. Mutants of complementation group 1 (P64 and P77) had significantly lower numbers of nodules compared to other mutant lines, and mutants of complementation group 2 could be classified into two groups. Lines of the first group, comprising P87, P88, P90, P91 and P93, showed an average of 627 nodules when grown without nitrate. Lines of the second group, comprising P89 and P94, had about 1000 nodules per plant (Fig. 2). Variability for nitrate tolerance was also detected and mutant lines could be classified into three groups. Mutants of the first group, comprising P64, P77 and P87, showed a decrease in nodule number with nitrate comparable to that of cv Frisson. In the second group, comprising P88, P89, P90, P91 and P93, no significant effect of nitrate on nodule number was detected. In the third group, comprising line P94, a significant increase in nodule number with nitrate was detected.

Table 4. Statistical significance of factor effects on nodule number and shoot dry matter per plant of pea cv Frisson, of supernodulating mutant P64 and the eight selected mutant lines grown in the presence of 0, 5, or 10 mM of nitrate.

Source of variation	df	Mean square	F value	Probability
Nodule number per plant				
Genotype	9	1776809	158	$<10^{-4}$
Nitrate concentration	2	21948	1.95	0.14
Genotype x nitrate	18	44668	3.98	$<10^{-4}$
Error	150	11233		
Shoot dry matter per plant				
Genotype	9	512110	331	$<10^{-4}$
Nitrate concentration	2	815955	53.5	$<10^{-4}$
Genotype x nitrate	18	20597	1.35	0.16
Error	150	15255		

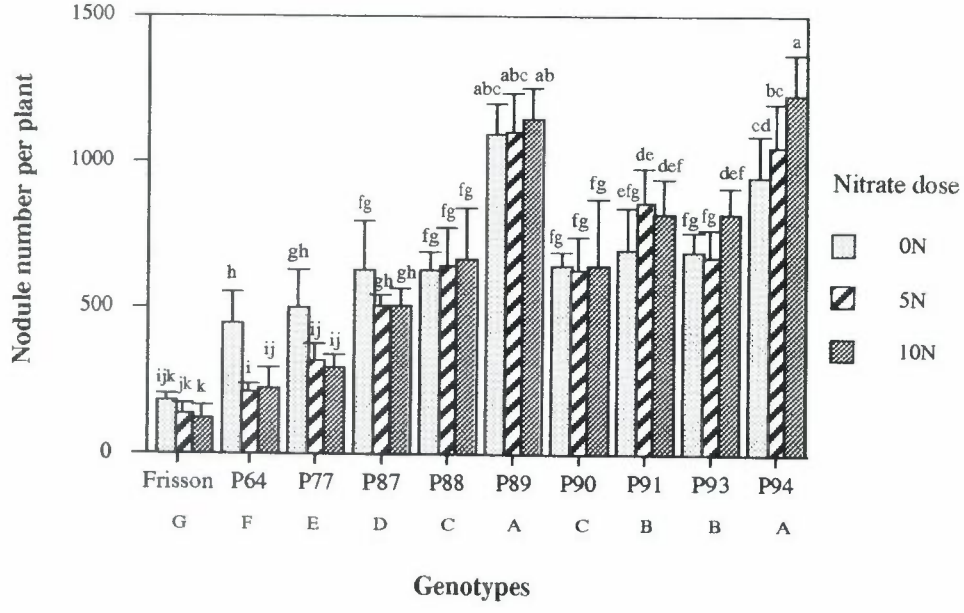

Figure 2. Number of nodules per plant at 30 days after inoculation on pea cv Frisson, supernodulating line P64, and eight selected mutants grown in the presence of 0 (0 N), 5 (5 N), or 10 (10 N) mM of nitrate. The bar represents one standard error. The same letter indicates means that are not significantly different according to the Newman-Keuls test at the 0.05 level.

A significant effect of genotype and of nitrate on shoot dry matter was detected through variance analysis, while no significant effect of the genotype x nitrate interaction was found (Table 5). Mutant lines all showed a lower dry matter accumulation in the shoot compared to cv Frisson when grown without nitrate (Fig. 3). However, there were differences between complementation groups and among lines of a same complementation group (Fig. 3). Mutants of complementation group 1 (P64 and P77) had significantly higher shoot dry matter than mutants of complementation group 2. Mutants of complementation group 2 could be classified into two groups. Mutants of the first group, comprising P87, P88, P89, P91, P93 and P94, had the lowest shoot dry matter (41% of the control line cv Frisson when grown without nitrate). Mutant P90 of the second group had shoot dry matter which was not significantly different from those of P64 and P88, with 75% of the control. Nitrate had a positive effect on all lines and a significant increase in dry matter accumulation was detected for cv Frisson and for all supernodulating lines (Fig. 3). This suggests that mutations did not affect assimilation of nitrate in the mutants.

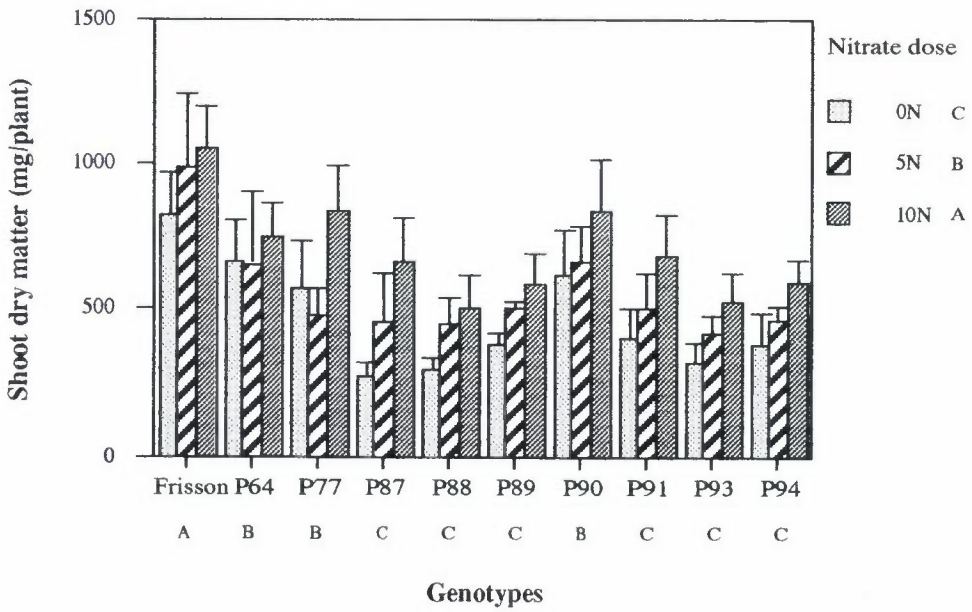

Figure 3. Shoot dry matter per plant at 30 days after inoculation on pea cv Frisson, supernodulating line P64, and eight selected mutants grown in the presence of 0 (0 N), 5 (5 N), or 10 (10 N) mM of nitrate. The bar represents one standard error. The same letter indicates means that are not significantly different according to the Newman-Keuls test at the 0.05 level.

Grafting experiments

In grafts between the nine supernodulating lines, P64, P77, P87, P88, P89, P90, P91, P93 and P94, and the parental line cv Frisson, the shoots of all the mutants induced a $Nod^{++}Nts$ phenotype in the roots of cv Frisson (Table 5). In contrast, roots of these mutants expressed a wild-type phenotype (normal nodulation in absence of nitrate, inhibition of nodulation by nitrate) when grafted with the shoot of cv Frisson (Table 5). This suggests that, in all selected lines, signals for the $Nod^{++}Nts$ character are located in the shoot.

Reciprocal grafts were made between the nine mutants and the nodulation mutants (Nod^{-} or $Nod^{+/-}$) P2, P54, P57 and DK24. Shoots of the $Nod^{++}Nts$ mutants never induced supernodulation, but the roots of the nodulation mutants kept their Nod^{-} or $Nod^{+/-}$ phenotype (Table 5). In contrast, the roots of the $Nod^{++}Nts$ mutants expressed a wild-type phenotype when grafted with shoots of nodulation mutants (Table 5). This confirms the root control of the non-nodulating character.

Table 5. Nodulation phenotype of grafts between cv Frisson, Nod^{++}Nts and nodulation (Nod$^-$ or Nod$^{+/-}$) mutant lines grown in the presence of 0 (0 N) or 10 (10 N) mM of nitrate. A wild-type's phenotype ([+]) means a cv Frisson-like nodulation (normal nodulation and inhibition of nodulation in the absence and presence of nitrate, respectively).

Root	Shoot			
	cv Frisson	Nod^{++}Nts lines	Nod$^-$ lines	Nod$^{+/-}$ lines
0 N				
cv Frisson	[+]	Nod^{++}	[+]	[+]
Nod^{++}Nts lines	[+]	Nod^{++}	[+]	[+]
Nod$^-$ lines	Nod$^-$	Nod$^-$	Nod$^-$	
Nod$^{+/-}$ lines	Nod$^{+/-}$	Nod$^{+/-}$		Nod$^{+/-}$
10 N				
cv Frisson	[+]	Nts	[+]	[+]
Nod^{++}Nts lines	[+]	Nts	[+]	[+]
Nod$^-$ lines	Nod$^-$	Nod$^-$	Nod$^-$	
Nod$^{+/-}$ lines	Nod$^-$	Nod$^-$		Nod$^-$

In a third experiment, reciprocal grafts were made between the super-nodulating mutants P64, P88, Nod3 and the wild-type line cv Frisson. Nodule number was measured at flowering stage. Self-grafts in cv Frisson, P64, P88 and Nod3 resulted in a 25.7, 26, 24.7 and 24.7% decrease in nodule number per plant respectively compared to the non-grafted plants (data not shown). Therefore, self-grafts were used as control plants in further graft comparisons. Super-nodulation was expressed in self-grafts of P64, P88 and Nod3 which showed a significant increase of 84, 139 and 367% respectively, in nodule number compared to cv Frisson (Fig. 4). Cv Frisson shoot on P64 or P88 roots and Nod3 shoot on cv Frisson roots had numbers of nodules non significantly different from cv Frisson self-grafts, while reciprocal grafts (P64 or P88 shoot on cv Frisson roots and cv Frisson shoot on Nod3 roots) had numbers of nodules non significantly different from P64, P88 and Nod3 self-grafts respectively (Fig. 4). This confirms the previously visually-determined shoot control of super-nodulation of P64 and P88 (Duc and Messager, 1989; this study) and the root control for Nod3 (Postma et al., 1988).

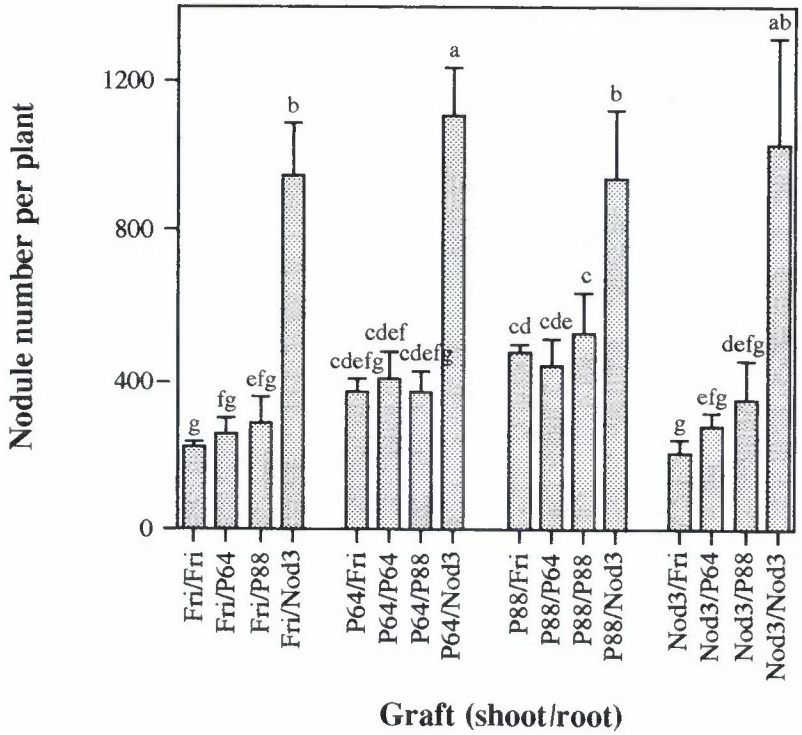

Figure 4. Number of nodules per plant at early flowering stage on grafts between cv Frisson and the supernodulating lines P64, P88 and Nod3. The same letter indicates means that are not significantly different according to the Newman-Keuls test at the 0.05 level.

Reciprocal grafts between supernodulating genotypes were without effect: (i) reciprocal grafts between P64 and P88 expressed numbers of nodules of the supernodulating shoot genotype. Grafts of P64 or P88 shoot on Nod3 roots had numbers of nodules non significantly different from cv Frisson shoot on Nod3 roots while the reciprocal graft had wild-type number of nodules (Fig. 4). This suggests the absence of additive effects of supernodulation characters in P64, P88 and Nod3.

In grafts with P64 or P77, no dissociation of the supernodulation and fasciation characters was detected. This is in agreement with the hypothesis that the same shoot signal controls supernodulation and fasciation in P64 and P77.

4. Discussion

Pea (*Pisum sativum* L.) is a legume species intensively used in genetic and molecular studies of the legume-*Rhizobium* symbiosis. A great number of genes specifically expressed in nodules (nodulin genes) have been characterized in pea (see Franssen et al., 1992 for a review). Mutagenesis programs have led to the selection of more than 100 mutant lines of pea (Jacobsen and Feenstra, 1984; Engvild, 1987; Postma et al., 1988; Kneen and LaRue, 1988; Duc and Messager, 1989; Kneen et al., 1990; Borisov et al., 1992) which have three classes of nodulation phenotype: (i) non nodulating (Nod^-); (ii) non-fixing (Nod^+Fix^-); and (iii) supernodulating and nitrate-tolerant nodulation ($Nod^{++}Nts$). Among these mutants, only a few $Nod^{++}Nts$ lines have been described (Jacobsen and Feenstra, 1984; Duc and Messager, 1989) and no complementation analyses of these mutants have been performed. In this paper, we describe the selection of eight new $Nod^{++}Nts$ mutants from pea cv Frisson. The mutation frequency for this character was one mutant for 1600 M_2 plants or one mutant line for 184 M_2 families. Duc and Messager (1989), selecting symbiotic mutants (Nod^-, Nod^+Fix^-, $Nod^{++}Nts$) from the same M_2 group, reported a 3-fold lower success rate. This frequency is also much higher than those observed in other cultivars of pea (Jacobsen and Feenstra, 1984; Engvild, 1987; Postma et al., 1988; Kneen and LaRue, 1988; Kneen et al., 1990; Borisov et al., 1992) or in soybean (Carroll et al., 1985; Carroll et al., 1986; Gremaud and Harper, 1989; Akao and Kouchi, 1992). These dissimilar rates could be due to differences in screening methods (in our experiment only Nts plants were selected), but also to the high mutation rate of one of the identified loci (seven of the eight lines belonged to the same complementation group). Such unusually high mutation rates have previously been described in pea symbiotic loci *a*, *d*, *j* (Duc and Messager, 1989), and *sym5* (Kneen and LaRue, 1984) and remains unexplained.

Complementation analyses of the eight selected mutants and the previously-described mutant P64 identified two genes involved in supernodulation and nitrate-tolerance in pea cv Frisson. As previously described in soybean (Carroll et al., 1985; Gremaud and Harper, 1989; Akao and Kouchi, 1992), common bean (Park and Buttery, 1988), faba bean (Duc, 1995) and pea (Jacobsen and Feenstra, 1984; Duc and Messager, 1989), Nod^{++} alleles were recessive against wild-type alleles. We propose to assign the gene codes *sym28* and *sym29* for the loci identified in P64 and P77, and P87, P88, P89, P90, P91, P93 and P94, respectively. Complementation analyses of cv Frisson mutant lines and the previously-described $Nod^{++}Nts$ mutant Nod3 from cv Rondo (Jacobsen and Feenstra, 1984) showed that Nod3 belonged to a different complementation group. This indicates that at least three genes involved in autoregulation of nodulation are available in pea. To our knowledge, only one gene has been

identified in soybean (Gresshoff, 1993b), common bean (Park and Buttery, 1988) and faba bean (Duc, 1995), which makes pea the legume species with the highest number of supernodulating mutants and identified genes.

Reciprocal grafting experiments showed that in all cv Frisson-derived mutant lines, Nod^{++} and Nts characters were controlled by the shoot genotype. This has been described in all supernodulating mutants so far (Delves et al., 1986, Buttery and Park, 1990; Cho and Harper, 1991; Duc, 1995; Francisco and Harper, 1995), except in the pea mutant line Nod3, where supernodulating phenotype is root-controlled (Postma et al., 1988). This indicates that regulation of nodulation is a complex process, at least in the pea species. More genetic variability for supernodulation character is necessary in other species to identify new genes and signals. The nature of these root and shoot signals and their relationships to the autoregulation process remains unknown. However, the associated supernodulation-shoot fasciation in *sym28* mutants suggests a defect in the hormonal balance of the plant, and the fact that the presence of *sym28* or *sym29* shoot does not modify Nod3 supernodulation (i.e., no "super-supernodulation" was observed) may signify that shoot signal(s) does not interact with root signal(s) in the autoregulation process. These mutant lines, now genetically-defined, would be useful tools to further study plant aspects in the regulation of nodulation and to identify these autoregulatory signals at the biochemical or molecular level.

Gene *sym29* identified in cv Frisson mutants showed phenotypic variation among mutant lines concerning nodule number, nitrate-tolerance of nodulation and shoot dry matter accumulation. This genetic material could also be of great agronomical interest, as already suggested in soybean where supernodulation was shown to be advantageous for the subsequent cereal crop (Song et al., 1995), or in pea as yield regulating factor (Sagan et al., 1993a).

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